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Phylogeographic analysis of two cold-tolerant plants with disjunct Lusitanian distributions does not support *in situ* survival during the last glaciation

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ABSTRACT

Aim We used a combination of modelling and genetic approaches to investigate whether *Pinguicula grandiflora* and *Saxifraga spathularis*, two species which exhibit disjunct Lusitanian distributions, may have persisted through the Last Glacial Maximum (LGM, *ca.* 21 ka) in separate northern and southern refugia.

Location Northern and eastern Spain and southwestern Ireland

Methods Palaeodistribution modelling using MAXENT was used to identify putative refugial areas for both species at the LGM, as well as to estimate their distributions during the Last Interglacial (LIG, *ca.* 120 ka). Phylogeographic analysis of samples from across both species' ranges was carried out using one chloroplast and three nuclear loci for each species.

Results The palaeodistribution models identified very limited suitable habitat for either species during the LIG, followed by expansion during the LGM. A single, large refugium across northern Spain and southern France was postulated for *P. grandiflora*. Two suitable regions, one in Northern Spain which corresponds to the eastern part of the species' present-day distribution in Iberia, as well another on the continental shelf off the west coast of Brittany, south of the limit of the British-Irish ice sheet, were identified for *S. spathularis*. Phylogeographic analyses indicated extremely reduced levels of genetic diversity in Irish populations of *P. grandiflora* relative to those in mainland Europe, but comparable levels of diversity between Irish and mainland European populations of *S. spathularis*, including the occurrence of private hapotypes in both regions.

Main conclusions Modelling and phylogeographic analyses indicate that *P. grandiflora* persisted through the LGM in a southern refugium, and achieved its current Irish distribution via northward dispersal after the retreat of the ice sheets. Although the results for *S. spathularis* are more equivocal, a similar recolonization scenario also seems the most likely explanation for the species' current distribution.

Keywords

Large-flowered butterwort, Last Glacial Maximum, Lusitanian flora, palaeodistribution modelling, *Pinguicula grandiflora*, phylogeography, refugia, *Saxifraga spathularis*, St. Patrick's cabbage.

INTRODUCTION

The present day distributions of many Northern Hemisphere temperate species are largely the result of the climatic fluctuations that occurred throughout the Pleistocene (1.8 Ma – 10 ka; Webb & Bartlein, 1992; Hewitt, 2003). During the extended glacial periods, many of these species persisted in climatically suitable refugia, usually south of the ice sheets, and recolonized formerly glaciated areas following the retreat of the ice during the interglacials, achieving their current distributions by the Holocene (10 ka – present; Taberlet *et al.* 1998; Hewitt 1999). In recent years, however, this simple "expansion-contraction" paradigm of species' persistence throughout the glacial periods has been challenged by palynological and phylogeographic evidence, which suggests that some species might have persisted in "cryptic" refugia further north than had previously been considered (Bennett and Provan 2008; Provan and Bennett 2008).

The distribution of the so-called "Lusitanian" flora, a group of about a dozen plant species that are found only in southern and western Ireland and northern Iberia, has long puzzled biogeographers. Some botanists proposed that this disjunct distribution was the result of persistence in separate northern and southern refugia during the ice ages, whilst others insisted that not even the hardiest of cold-tolerant plants could survive through the Last Glacial Maximum (LGM; *ca*. 21 ka) *in situ* (Forbes 1846; Reid 1913; Praeger 1939; Webb 1983). Recently, the first phylogeographic study on a Lusitanian plant species, the heath *Daboecia cantabrica*, suggested that the species had been confined to southern refugia during the LGM, and had achieved its Irish distribution following the deglaciation (Beatty and Provan 2013). *D. cantabrica* has minute seeds conducive to dispersal, and it may be that its glacial history is not typical of the Lusitanian flora as a whole, particularly for cold-tolerant species which may have been more likely to survive in northern refugia.

In the present study, we have used a combination of palaeodistribution modelling and phylogeographic analysis to determine whether two of the more cold-tolerant Lusitanian plant species, *Pinguicula grandiflora* Lam. (Large-flowered butterwort) and *Saxifraga spathularis* Brot. (St. Patrick's cabbage), might have survived in northern refugia during the LGM. *S. spathularis* has a typical Lusitanian distribution, being found only in northwestern Spain and southwest Ireland, whilst *P. grandiflora* has a wider distribution in Spain, being found across the north of the country and into the Pyrenees, as well as sporadically in the French and Italian alps, and in southwest Ireland (Figures 1a and 1b). If either species had persisted in northern refugia, Irish populations would exhibit comparable genetic diversity to those from Spain whilst harbouring unique genotypes, the two key phylogeographic signatures of long-term persistence (Provan and Bennett 2008).

MATERIALS AND METHODS

Sampling and DNA extraction

Samples were obtained for both species from across their distribution ranges either through collection in the field or from herbarium collections (See Appendices S1a and S1b for details). In total, between 149 and 160 samples of *Pinguicula grandiflora* from 38 locations, and between 161 and 176 samples of *Saxifraga spathularis* from 41 locations were analysed. Differences in sample numbers were due to lack of amplification in several individuals at one or more loci. DNA was extracted from field-collected material using a modified CTAB protocol (Doyle & Doyle 1987) and from herbarium samples using the Qiagen DNeasy kit.

Palaeodistribution modelling

Palaeodistribution modelling was carried out to determine suitable climate envelopes for both species during the last interglacial (LIG, *ca*. 120 ka) and at the last glacial maximum (LGM, *ca*. 21 ka) using the maximum entropy approach implemented in the MAXENT software package (V3.3.3; Phillips *et al*. 2006). Species occurrence data between 1950 and 2000 (269 and 385 occurrences for *P. grandiflora* and *S. spathularis* respectively) were downloaded from the Global Biodiversity Information Facility data portal (www.gbif.org). Current-day climatic data (1950-2000; Hijmans *et al.* 2005) at 2.5 minute resolution were clipped to the approximate distribution area of the species (i.e. Western Europe 13 °W to 10 °E, and 35°N to 60°N) to reduce potential problems associated with extrapolation. Models were generated using cross-validation of ten replicate runs under the default MAXENT parameters. Model performance was assessed based on the area under the receiver operating characteristic curve (AUC). Models were projected onto reconstructed climate data for the LGM (two models: CCSM and MIROC) and the LIG (WorldClim www.worldclim.org). Outputs from the two

LGM models were averaged to give a single consensus model. To identify areas where the model has extrapolated beyond current climatic conditions, which could lead to unreliable predictions, we carried out a multivariate environmental similarity surfaces (MESS) analysis (Elith *et al.* 2010) in MAXENT.

DNA sequencing

Sequence data were obtained from one chloroplast locus (the *trn*L-*trn*F intergenic spacer for *P. grandiflora* and the *trn*S-*trn*G intergenic spacer for *S. spathularis*) and three anonymous single-copy nuclear loci for each species (details and primer sequences are given in Appendix S2). Primers to amplify anonymous single-copy nuclear DNA loci were developed using the ISSR-cloning method described in Beatty *et al.* (2010). For herbarium samples from which the complete chloroplast product could not be amplified in a single PCR, the region was amplified as two or three overlapping fragments using internal primers (Appendix S3). PCR was carried out on a MWG Primus thermal cycler (Ebersberg, Germany) using the following parameters: initial denaturation at 94 °C for 3 min followed by 45 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C (52 °C for *S. spathularis* trnS-G intergenic spacer) for 30 s, extension at 72 °C for 1 min and a final extension at 72 °C for 5 min. PCR was carried out in a total volume of 20 µl containing 200 ng genomic DNA, 10 pmol of each primer, 1x PCR reaction buffer, 200 µM each dNTP, 2.5 mM $MgCl_2$ and 0.5 U GoTaq Flexi DNA polymerase (Promega, Sunnyvale, CA). Five µl PCR product were resolved on 1.5% agarose gels and visualised by ethidium bromide staining, and the remaining 15 µl were EXO-SAP purified and sequenced in both directions using the BigDye sequencing kit (V3.1; Applied Biosystems) and run on an AB 3730XL DNA analyser (Life Technologies; Carlsbad, California, USA).

Phylogeographic analysis

DNA sequences were aligned in BIOEDIT (V7.0.9.0; Hall, 1999). For the single-copy nuclear loci, haplotypes were resolved for individuals exhibiting two or more heterozygous positions using the PHASE program (V2.1; Stephens & Donnelly 2003) implemented in the DnaSP software package (V5.10; Librado & Rozas 2009). *Pinguicula grandiflora* is tetraploid (Heslop-Harrison 2004), but to avoid difficulties with estimation of allele dosage, all heterozygotes were scored as 50:50 i.e. similar to treatment of diploid loci. Given the extremely low levels of heterozygosity observed in Irish populations relative to continental European populations, this should not unduly affect our conclusions regarding the glacial history of the species. Potential recombination was assessed using the Hudson & Kaplan (1985) test in DnaSP. Median-joining networks for all loci were constructed using the NETWORK software package (V4.5.1.6; www.fluxus-engineering.com). Any reticulations in the networks were broken following the rules described in Pfenninger & Posada (2002). Levels of haplotype diversity (H) and nucleotide diversity (π) were calculated using DnaSP. To account for differences in sample sizes, particularly in the case of *S. spathularis*, levels of haplotype richness (R_h) were calculated using HAPLOTYPE ANALYSIS (V1.05; Eliades & Eliades 2009).

To assess potential geographical structuring of genetic variation in continental European populations associated with persistence in multiple glacial refugia, we performed a spatial analysis of molecular variance (SAMOVA) using the SAMOVA software package (V1.0; Doupanloup *et al.* 2002) for each of the eight data sets. The program uses a simulated annealing approach based on genetic and geographical data to identify groups of related populations. The program was run for 10,000 iterations for $K = 2$ to 10 groups from 200 initial conditions, and the most likely structure was identified using the maximum value of Φ_{CT} that did not include any groups of a single population.

RESULTS

Palaeodistribution modelling

For all models, AUC values were high (*P. grandiflora* mean AUC = 0.944 SE = 0.008; *S. spathularis* mean AUC = 0.973 SE = 0.005). Palaeodistribution modelling of species distributions suggested far more restricted ranges during the LIG compared to current distributions, particularly for *S. spathularis* (Figures 1c and 1d). The models suggested that both species had larger potential distribution ranges during the LGM. For *P. grandiflora*, extensive regions of suitable habitat coincided largely with the species' current distribution in continental Europe, with additional areas in southern France where the species is currently absent (Figure 1e). Two main areas of suitable habitat during the LGM were identified for *S. spathularis*, one in Northern Spain which corresponds to the eastern part of the species' present-day distribution in Iberia, as well another on the continental shelf off the west coast of Brittany, south of the limit of the British-Irish ice sheet (Figure 1f).

Phylogeographic analysis

Between three (*trn*L-F) and twelve (Pg-C01 and Pg-F10) haplotypes were found in the four loci analysed in *P. grandiflora* (Table 1 and Figure 2). Populations from continental Europe harboured far higher levels of diversity than those from Ireland, with total of 37 haplotypes across the four loci, including 31 private haplotypes, compared to six haplotypes (one private) in Irish populations. The frequency of private alleles ranged from 0.09 (Pg-C02) to 0.46 (trnL-F) in continental Europe, whilst the sole private allele in Irish populations, at locus Pg-F10, was only found at a frequency of 0.01. Levels of haplotype richness (R_h), haplotype diversity (H), and nucleotide diversity (π) ranged from 2.000 (*trn*L-F) to 9.087 (pg-F10), from 0.179 (Pg-C01) to 0.606 (*trn*L-F), and from 0.0007 (Pg-F10) to 0.0030 (Pg-F02)

172 respectively in continental European populations, and from zero (*trn*L-F and Pg-C02) to
173 0.480 (Pg-F02), from zero (*trn*L-F and Pg-C01) to 1.000 (Pg-F02 and Pg-F10), and from zero
174 (*trn*L-F and Pg-C01) to 0.0036 (Pg-F02) respectively in Irish populations.
175 Evidence for recombination was detected at all three nuclear loci analysed in *S.*
176 *spathularis*. Consequently, subsequent analyses were carried out using only the largest non-
177 recombining portion of each locus (see Appendix S3 for details). The chloroplast *trn*S-G
178 intergenic spacer region exhibited eleven haplotypes in Spain (R_h = 10.000, H = 0.872, π =
179 0.0020), but only a single haplotype in Ireland (Table 1 and Figure 3). For three nuclear loci,
180 values for R_h, H and π ranged from 3.000 (Ss-G04) to 5.000 (Ss-C02), from 0.238 (Ss-G04)
181 to 0.557 (Ss-G07), and from 0.0026 (Ss-G04) to 0.0076 (Ss-C02) respectively in Spain, and
182 from 1.000 (Ss-G04) to 4.491 (Ss-C02), from 0.162 (Ss-G04) to 0.581 (Ss-C02), and 0.0017
183 (Ss-G04) to 0.0084 (Ss-C02) respectively in Ireland. All four loci exhibited private
184 haplotypes in Spain, whilst two of the four (Ss-C02 and Ss-G07) had private haplotypes in
185 three of the Irish populations studied.
186 For both species, none of the SAMOVA analyses (four loci for each species) indicated
187 any obvious geographical structuring of genetic variation (data not shown).

DISCUSSION

Phylogeographic studies are increasingly being used in conjunction with palaeodistribution modeling to provide insights into the response of species to the glacial periods of the late Pleistocene (for reviews see Chan *et al.* 2011; Alvaredo-Serrano & Knowles 2014). It is important, however, to appreciate the potential pitfalls and problems of such modeling approaches, particularly for species with restricted and/or limited distributions, as is the case for the Lusitanian flora. The main drawback with ecological niche models based on correlative approaches is that these approaches assume species / environment equilibrium, a condition which is frequently violated when examining range-shifts such as those associated with the glaciations (Menke *et al.* 2009; Elith *et al.* 2010). The incorporation of multivariate environmental similarity surface (MESS) methods (Elith *et al.* 2010) into the most recent versions of the MAXENT modeling software package (V3.3.2 onwards) allows identification of areas in the model where extrapolation is greatest, and consequently where prediction may be less reliable e.g. areas that lie under the ice sheets at the LGM (Figure S3, Additional Supporting Material). When applied to the LIG and LGM models for both of our study species, the only modeled area that was associated with strongly negative (i.e. less than -10) MESS values, and consequently may reflect unreliable prediction, was the northeastern part of the modeled LGM range for *P. grandiflora* in eastern Aquitaine and the Midi-Pyrénées regions of France. The apparently larger distributions for both species at the LGM relative to the LIG may reflect the fact that they are cold-tolerant to some degree (Webb 1983), and many cold-tolerant species have been suggested to have larger distributions during glacial periods (reviewed in Bennett & Provan 2008; Stewart *et al.* 2010). Nevertheless, although the observed patterns of genetic variation in *P. grandiflora* are consistent with the occurrence of a single large refugium (see below), caution should be exercised when trying to make

inferences on past ranges for species with restricted distributions (Elith *et al.* 2010). Although all models had high AUC values, which generally indicates good predictive power, these values tend to be inflated for species which occupy a limited part of the area analyzed, as typified by species that exhibit disjunct distributions where large areas are unoccupied.

Pinguicula grandiflora – the classic southern refugium paradigm

Both the palaeodistribution modelling and the phylogeographic evidence indicate that *P. grandiflora* persisted throughout the LGM in a southern refugium and recolonized Ireland following the retreat of the ice sheets. Unlike *Daboecia cantabrica*, the only other plant with a Lusitanian distribution on which phylogeographic analysis has been carried out (Beatty & Provan, 2013), and which survived the LGM in two southern refugia, the lack of geographical structuring of genetic variation in continental European populations of *P. grandiflora* suggests the occurrence of a single refugium. This is consistent with the large, mostly continuous area of suitable habitat at the LGM indicated by the palaeodistribution modelling. The extremely low levels of genetic diversity observed in Irish populations, coupled with the occurrence of only a single, low frequency private haplotype, are indicative of the founder effects associated with postglacial recolonization (Hewitt 1999; Provan & Bennett 2008). This is in contrast to Webb (1983), who could conceive no mechanism whereby *P. grandiflora* could have reached Ireland from the Pyrenees, and concluded that the species must have persisted *in situ* since the interglacial. Such persistence seems unlikely, since although hibernacula can withstand low temperatures, rosettes are extremely susceptible to frost (Heslop-Harrison 1962; Grace 1987). Seeds of *P. grandiflora* are minute and dust-like (as are those of *D. cantabrica*), a feature which could facilitate long-distance dispersal following the retreat of the ice after the LGM.

Saxifraga spathularis – evidence for persistence in a northern refugium?

Whilst *P. grandiflora* exhibits the classic "southern richness vs. northern purity" pattern of genetic diversity consistent with survival in southern refugia (Hewitt 1999), the distribution of genetic diversity in *S. spathularis* initially appears incompatible with such a scenario. Levels of haplotype diversity in Irish populations are comparable to those found in Spain for the three nuclear loci studied, and private haplotypes are present in Ireland at two of these loci, although after rarefaction to account for differences in sample size, levels are lower for all three loci in Ireland. This is in contrast, however, to the data from the chloroplast *trn*S-G intergenic spacer, which suggest an extreme bottleneck in Irish populations, effectively ruling out the possibility that Iberian populations might have originated in a northern refugium. The discrepancy between the chloroplast and nuclear markers is most likely due to the smaller effective population size of the haploid, uniparentally inherited chloroplast genome, which will be more susceptible to effects of genetic drift during the population fluctuations associated with climatic changes during the Pleistocene.

Despite the comparable levels of nuclear diversity and occurrence of private haplotypes, it seems unlikely that *S. spathularis* persisted through the LGM in separate northern and southern refugia, as suggested by the palaeodistribution model, since this would lead to phylogenetic structuring which would be reflected geographically. The opposite is apparent for locus Ss-G07, where the two private haplotypes found in Ireland, depicted in yellow and white, are two of the most phylogenetically divergent haplotypes, being separated by four mutations. It is possible that these represent localized recent mutations, since they are present at very low frequencies. Alternatively, it might be that the lower number of plants analyzed from Spain means that these haplotypes are present there, but were simply not sampled.

The patterns of genetic variation observed in *S. spathularis* are similar to those reported for *Meconopsis cambrica*, an herbaceous species with a disjunct distribution similar to that

exhibited by the Lusitanian flora, being found in Northern Spain and the Massif Central, as well as in north Devon, Somerset, Wales and Ireland (Valtueña *et al.* 2012). Populations of *M. cambrica* from Wales shared a single chloroplast haplotype with samples from northeastern Spain, but also displayed comparable levels of nuclear diversity to some continental European populations, including private AFLP alleles. It was suggested that these populations might have originated from a separate northern refugium, but not as far north as mainland Britain. Unlike in *M. cambrica*, however, *S. spathularis* populations are not separated into "northern" and "southern" groups, and Irish populations most likely shared a refugial area with present-day Spanish populations. The lack of correspondence with the palaeodistribution model, which did suggest the potential existence of separate northern and southern refugial areas, is probably a consequence of the aforementioned problems associated with extrapolating ranges of species with restricted distributions, as is the case with *S. spathularis* (Elith *et al.* 2010). This could also explain the extremely low levels of predicted suitable habitat during the LIG.

An alternative explanation for the occurrence of private haplotypes in Ireland could be introgression from the congeneric *S. hirsuta*. Both species occur sympatrically in Cos. Cork and Kerry, where they hybridize to form *S.* x *polita* (Webb, 1951). Although *S. hirsuta* is much less common than *S. spathularis*, introgression of species-specific SNP alleles from the former into the latter has been observed, even in populations of *S. spathularis* from Co. Galway where *S. hirsuta* is now absent (G.E. Beatty & J. Provan, unpublished results). Such introgression is consistent with the high levels of recombination detected at all three nuclear loci, but would not affect the chloroplast genome. Unfortunately, though, it was not possible to amplify the two orthologous loci of Ss-C02 and Ss-G07 in *S. hirsuta* to determine whether the alleles had been introgressed into *S. spathularis* from its congener.

CONCLUSIONS

The findings of the present study, together with the only previously published phylogeographic study on a member of the Lusitanian flora, *Daboecia cantabrica* (Beatty & Provan, 2013), suggest that the original theory of persistence in separate northern and southern refugia cannot explain these species' puzzling distribution. Both *Pinguicula grandiflora* and *Saxifraga spathularis*, like *D. cantabrica*, are likely to have persisted through the LGM in southern refugia and colonized Ireland after the retreat of the ice sheets. Nevertheless, the differing refugial histories of the three species suggest that no one scenario can account for the present-day distribution of the Lusitanian flora, and that several southern refugia facilitated these species' survival during the LGM.

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BIOSKETCHES

Gemma Beatty is a Postdoctoral Research Fellow at Queen's University Belfast. Her PhD research compared how postglacial recolonization and range-edge effects have shaped the genetic diversity of several northern hemisphere Monotropoideae species. She is interested in using genetic approaches to study the effects of past and present climate change on the distribution ranges of natural populations, and the various factors that determine these ranges.

Jim Provan is a Reader in Evolutionary Genetics at Queen's University Belfast. His research interests focus on how genetic variation is distributed across species ranges, and on the effects of past, present and future climate change on levels and patterns of intraspecific diversity.

Author contributions: JP conceived the study; both authors collected and analysed the data, and wrote the manuscript.

Editor: Hans-Peter Comes

Table 1 Diversity statistics by region. *N* – number of individuals; *h* – number of haplotypes observed (number of private haplotypes in parentheses); $Freq_p$ – frequency of private haplotypes; R_h – Haplotype richness; *H* – haplotype diversity; π - nucleotide diversity.

Species	Locus	Europe						Ireland						GenBank
		N	*h*	$Freq_p$	R_h	*H*	π	*N*	*h*	$Freq_p$	R_h	*H*	π	Accessions
P. grandiflora	*trn*L-F	92	3 (2)	0.46	2.000	0.606	0.0010	68	1 (0)	-	-	-	-	
	Pg-C01	180	12 (11)	0.09	8.951	0.179	0.0011	140	1 (0)	-	-	-	-	
	Pg-F02	180	11 (9)	0.14	8.042	0.455	0.0030	118	2 (0)	-	1.000	0.480	0.0036	
	Pg-F10	166	11 (9)	0.10	9.087	0.190	0.0007	136	2 (1)	0.01	1.000	0.015	0.0001	
S. spathularis	*trn*S-G	39	11 (10)	0.85	10.000	0.872	0.0020	134	1 (0)	-	-	-	-	
	Ss-C02	78	6 (2)	0.21	5.000	0.532	0.0076	244	8 (4)	0.02	4.491	0.581	0.0084	
	Ss-G04	76	4 (2)	0.03	3.000	0.238	0.0026	274	2 (0)	-	1.000	0.162	0.0017	
	Ss-G07	80	5 (3)	0.06	4.000	0.557	0.0051	272	4 (2)	0.02	2.257	0.500	0.0048	

Figure Legends

Figure 1 Present-day distributions of (a) *Pinguicula grandiflora*, and (b) *Saxifraga spathularis* (shaded; based on Webb 1982 and the Global Biodiversity Information Facility [data.gbif.org]) in Europe. Palaeodistribution models for (c,e) *P. grandiflora*, and (d,f) *S. spathularis* during the Last Interglacial (*ca.* 120 ka) and the Last Glacial Maximum (*ca.* 21 ka) repectively. Darker areas in (c-f) show most suitable modelled habitat. The limits of the British-Irish ice sheet (after Sejrup *et al.* 2005) and Alpine and Pyrenean glaciers (after Ehlers & Gibbard 2004) at the LGM are also indicated.

Figure 2 Haplotype distributions for (a) chloroplast *trn*L-F intergenic spacer, (b) nuclear Pg-C01 region, (c) nuclear Pg-F02 region, and (d) nuclear Pg-F10 region for *Pinguicula grandiflora*. Pie chart sizes are approximately proportional to sample size, with the smallest circles representing $N = 1$ (chloroplast locus) or $N = 2$ (nuclear loci) and the largest representing $N = 8$ (chloroplast locus) or $N = 16$ (nuclear loci). In the haplotype networks, black circles represent unique haplotypes found in a single individual, and open diamonds indicate missing (unsampled) haplotypes.

Figure 3 Haplotype distributions for (a) chloroplast *trn*S-G intergenic spacer, (b) nuclear Ss-C02 region, (c) nuclear Ss-G04 region, and (d) nuclear Ss-G07 region for *Saxifraga spathularis*. Pie chart sizes are approximately proportional to sample size, with the smallest circles representing $N = 1$ (chloroplast locus) or $N = 2$ (nuclear loci) and the largest representing $N = 8$ (chloroplast locus) or $N = 16$ (nuclear loci). In the haplotype networks, black circles represent unique haplotypes found in a single individual, and open diamonds indicate missing (unsampled) haplotypes.

SUPPORTING INFORMATION

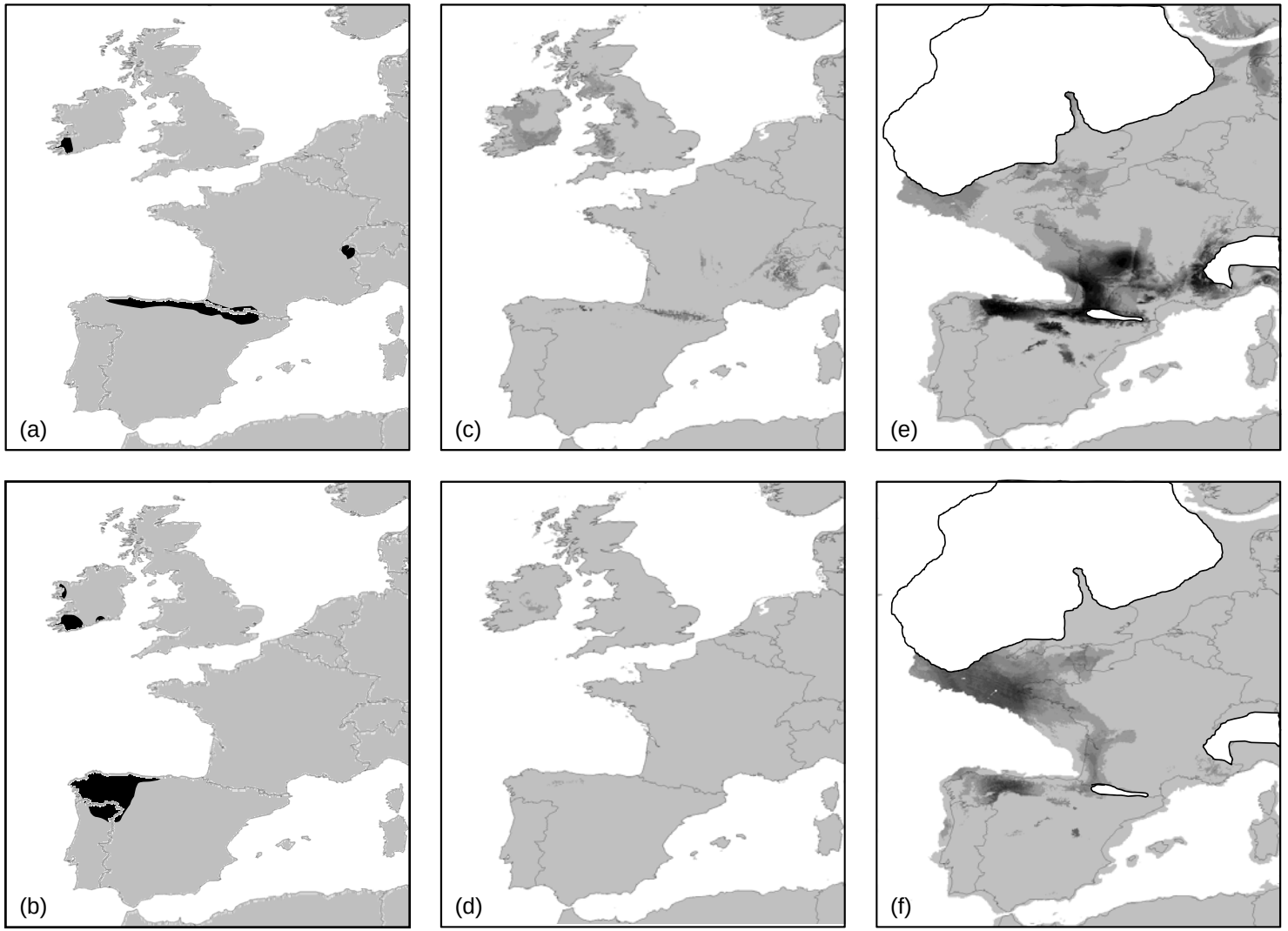
Additional Supporting Information may be found in the online version of this article:

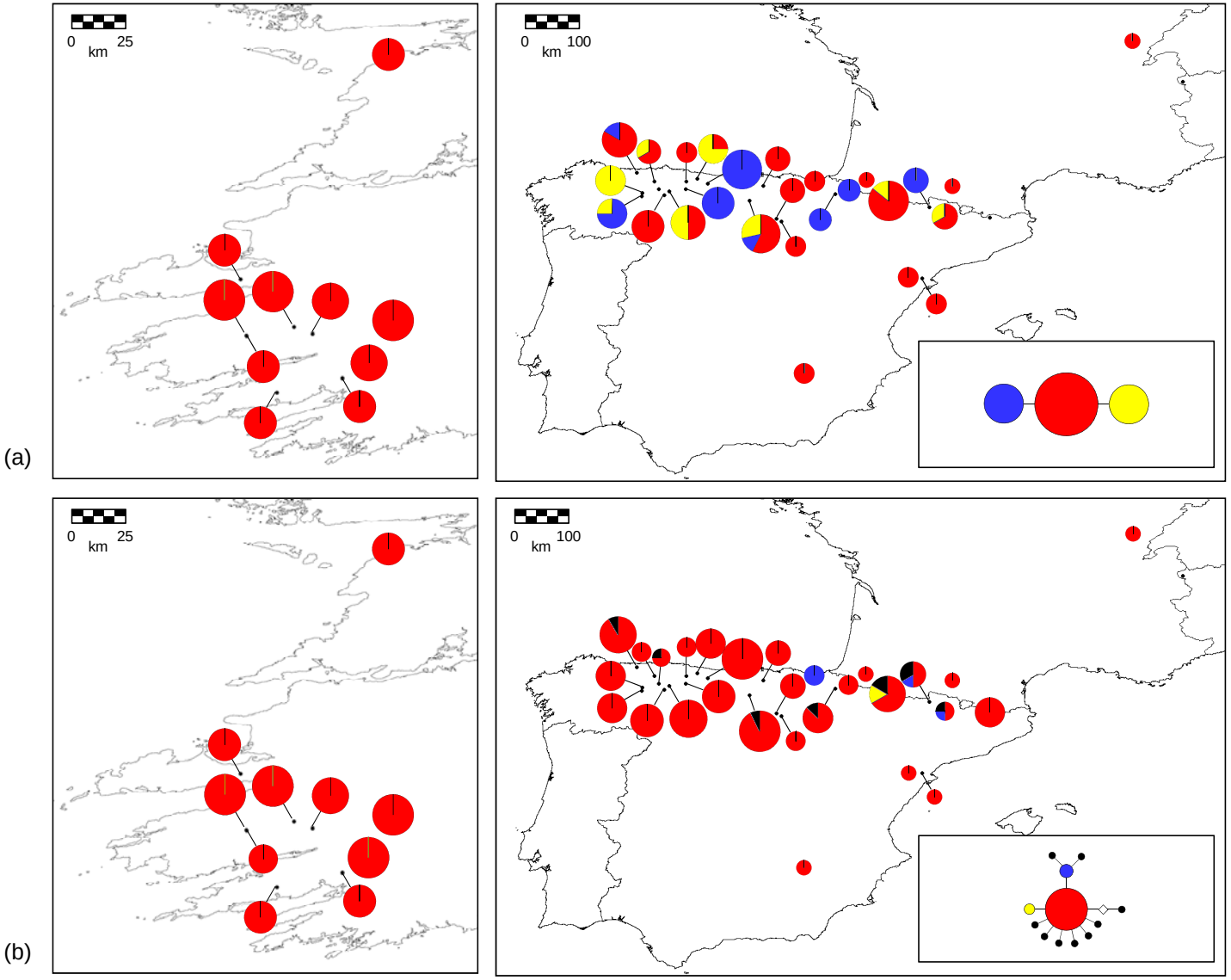
Appendix S1a Samples of *Pinguicula grandiflora* analysed

Appendix S1b Samples of *Saxifraga spathularis* analysed

Appendix S2 PCR / sequencing primers used in this study.

Figure S3 Results of the multivariate environmental similarity surfaces (MESS) analysis for (a) LIG, and (b) LGM models. Darker blue areas highlight regions with more highly negative MESS values, indicating areas where the model has extrapolated beyond current climatic conditions, which could lead to unreliable predictions.


(a)
(c)
(e)
(b)
(d)
(f)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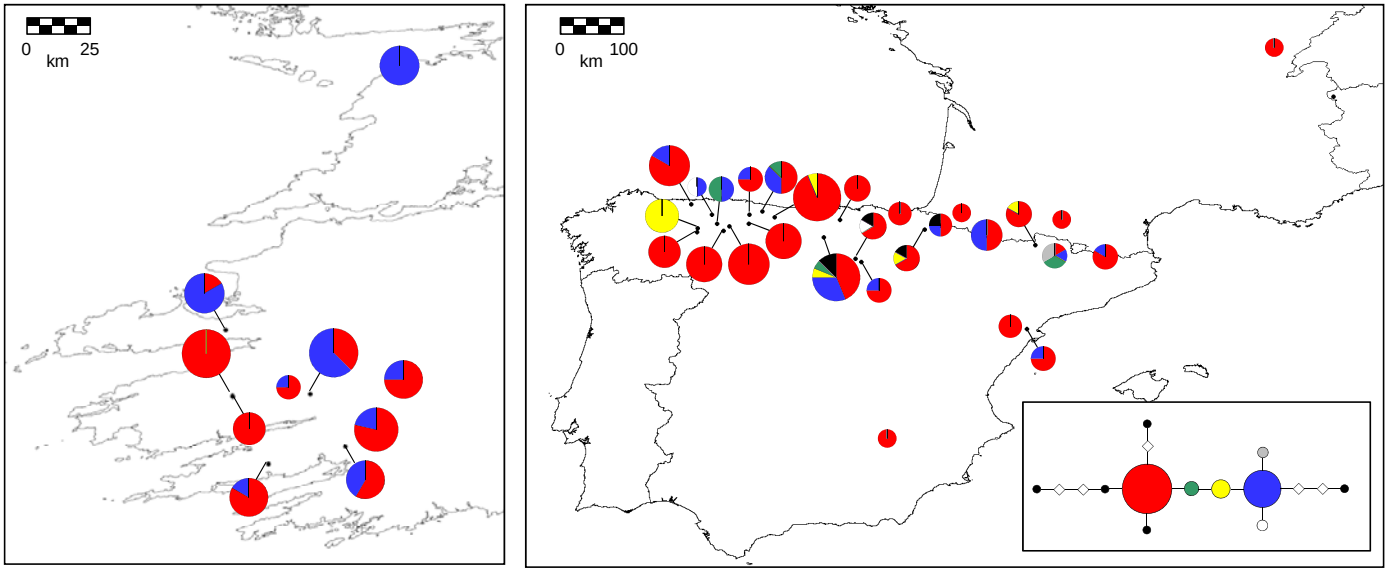
0 km 25
0 km 100
(b)
0 km 25
0 km 100

(c)



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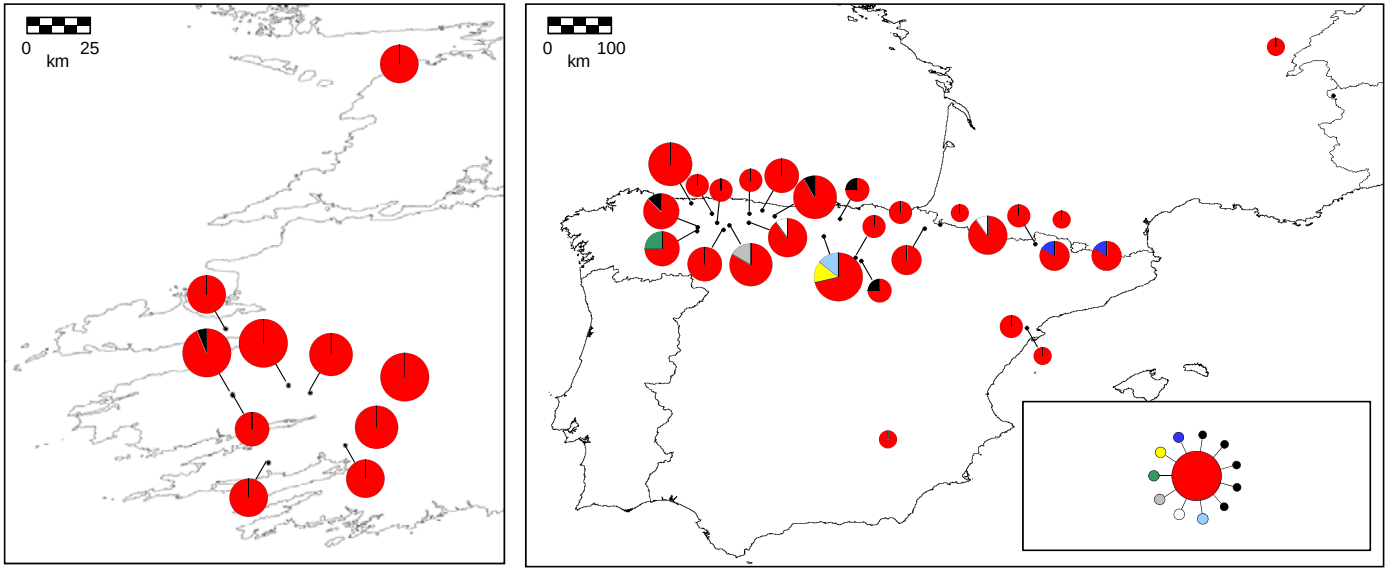
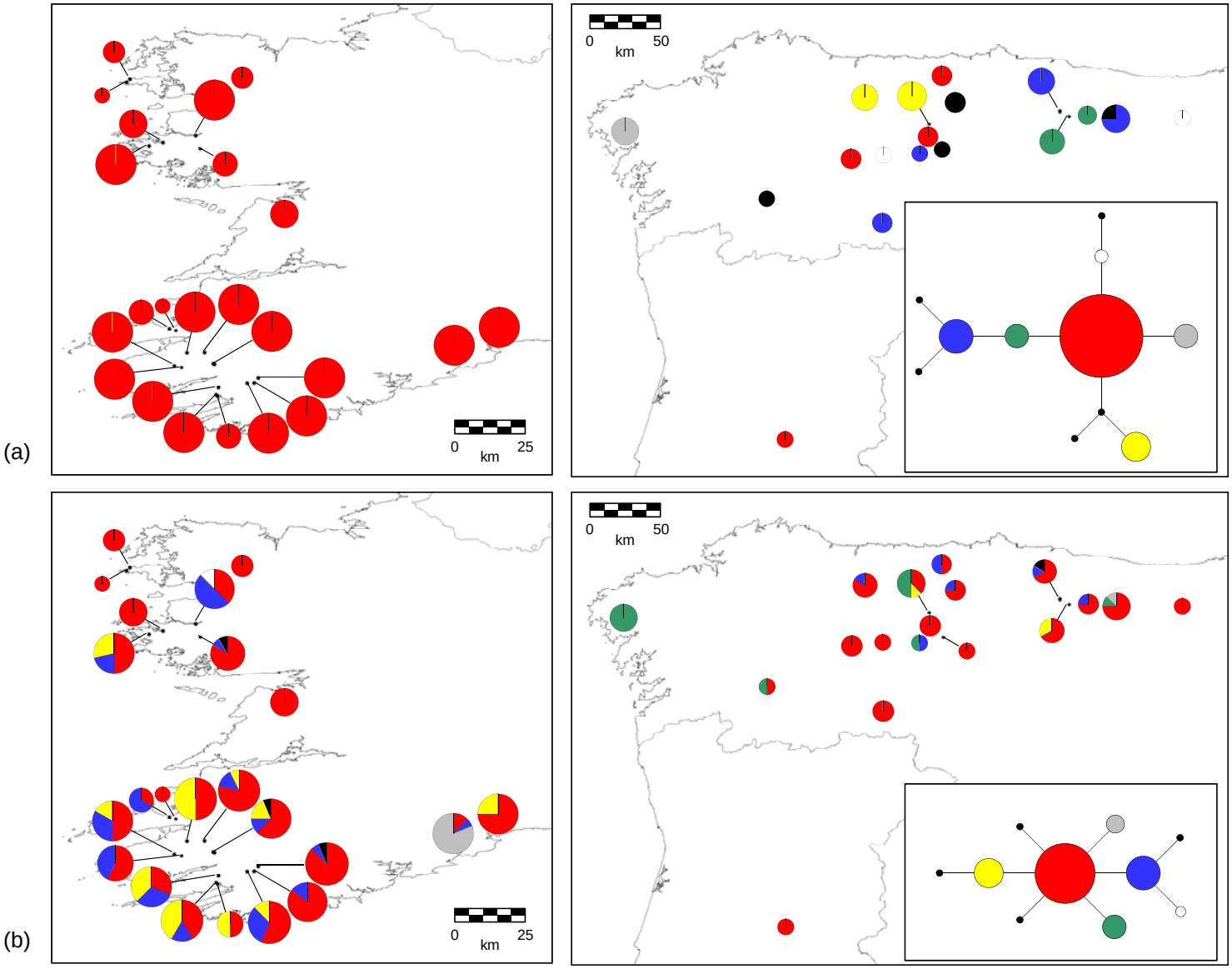
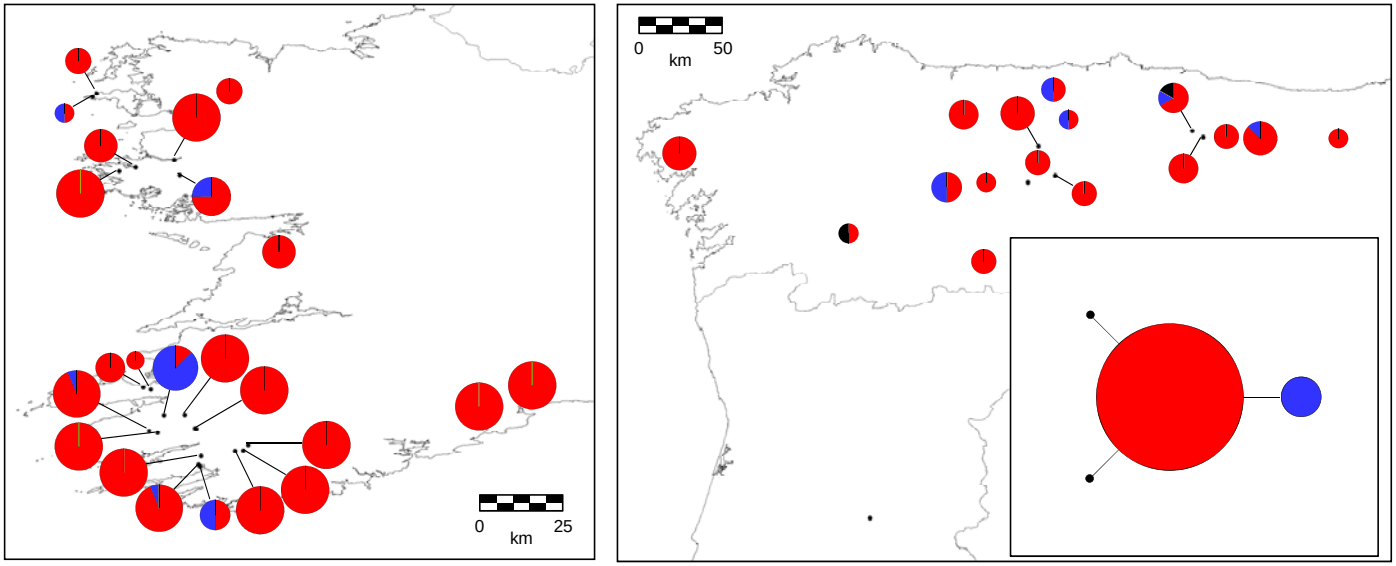
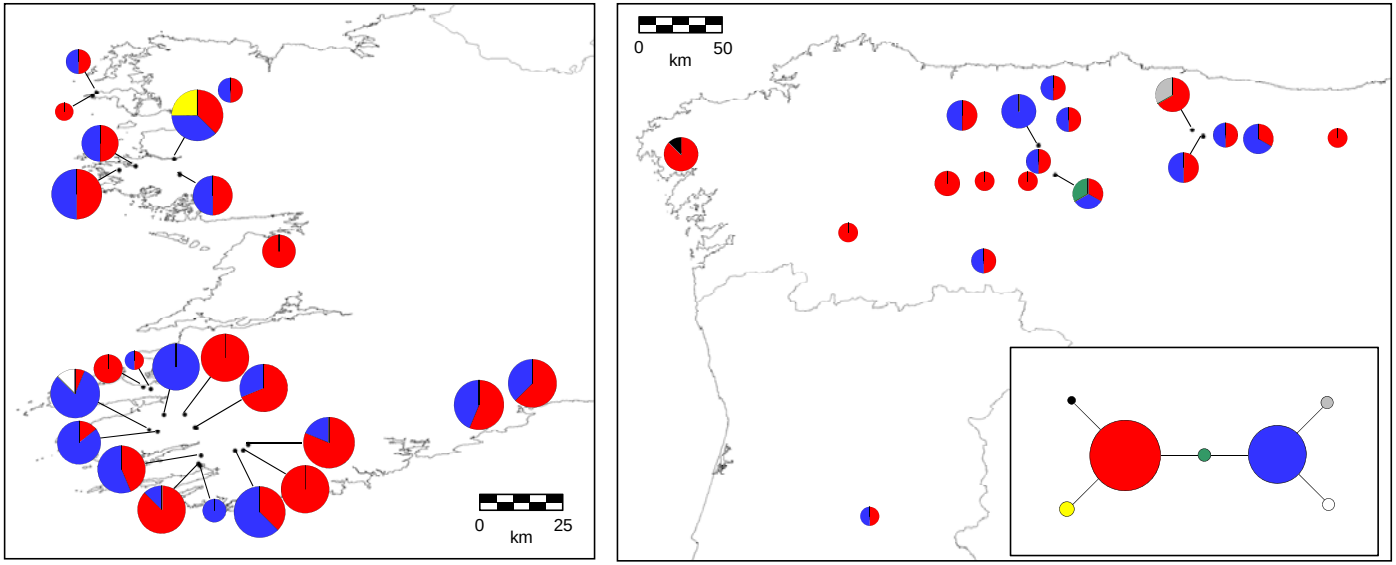



(a)
0
km
50
0
km
25
(b)
0
km
50
0
km
25

(c)



(d)



Appendix S1a Samples of *Pinguicula grandiflora* analysed

Country	Location	Code*	Lat (N)	Long (W)	*n*			
					*trn*L-F	Pg-C01	Pg-F02	Pg-F10
France	Pyrénées-Atlantiques	G-00308102	43.2503	0.8794	1	1	1	1
	Bagnère-de-Luchon	G-00308101	43.1183	-1.2436	1	1	1	1
	Près Lajoux	G.00308100	46.7392	-5.7289	1	1	1	1
Ireland	Lough Cloon 1		51.9573	9.8530	6	6	4	5
	Lough Cloon 2		51.9547	9.8525	8	8	8	8
	Gap of Dunloe		51.9892	9.6562	8	8	2	8
	Galway Bridge		51.9628	9.5793	7	8	8	8
	Gougane Barra		51.8333	9.3450	7	8	7	8
	Killarney National Park		52.0155	9.2461	8	8	6	7
	Priest’s Leap		51.7781	9.4564	6	6	6	6
	Healy Pass		51.7152	9.7270	6	6	6	6
	Slieve Mish		52.1881	9.8773	6	6	6	6
	Burren		53.1258	9.2699	6	6	6	6
Spain	Valdés	FCO-15016	43.46	6.56	6	6	6	6
	Villariño del Sil	LEB-15742	42.8737	6.4289	4	4	4	4
	Puerto de Leitariegos	LEB-13532	42.9637	6.4252	4	4	4	4
	Yernes y Tameza	FCO-23468	43.24	6.12	3	2	1	2
	Puerto de la Ventana	SEV-68044	43.0568	6.0106	-	2	2	2
	Mirantes de Luna	LEB-3760	42.9099	5.88	5	5	5	4
	Branillín	LEB-63747	43.0044	5.7606	6	6	6	6
	Puerto de San Isidro	LEB-62112	43.0541	5.3502	5	5	5	5
	Cangas de Onís	FCO-26683	43.24	5.34	2	2	2	2
	Covadonga	SEV-25704	43.3088	5.0544	4	4	4	4
	Cabrales	FCO-13569	43.19	4.80	8	7	8	6
	Valdelateja	SEV-18909	42.7743	3.7687	7	6	8	7
	Merindad de Montija	SALA-103945/104063	43.13	3.43	3	3	3	2

Appendix S1a (continued)

Country	Location	Code*	Lat (N)	Long (W)	*n*			
					*trn*L-F	Pg-C01	Pg-F02	Pg-F10
Spain	Valgañón	SALA-100469	42.32	3.10	3	3	3	2
	Ezcaray	SALA-100470	42.25	2.98	2	2	2	2
	Riopar	SEV-53522	38.499	2.417	2	1	-	1
	Aia	SALA-88654	43.23	2.17	2	2	2	2
	Valle del Roncal	SEV-91312	42.9265	1.6515	4	4	3	3
	Roncesvalles	SEV-224332	43.0135	1.3157	2	2	2	-
	Sallent de Gállego	FCO-7678/SEV-69895	42.79	0.33	7	6	4	5
	Beceite	BC-905331	40.8311	-0.1796	2	1	2	2
	Roquetes	BC-905330	40.8207	-0.5018	2	1	2	1
	Valle de Arán	SALA-10903	42.61	-0.68	3	3	3	2
	Arcalis	BC-912895	42.3542	-1.0838	3	2	3	3
	Queralbs	SEV-92951	42.3505	-2.1710	-	4	3	3
					160	160	149	151

* Herbarium codes: G - Université de Genève; FCO - Universidad de Oviedo Herbario; LEB - Universidad de León Herbario; SEV - Universidad de Sevilla Herbario; SALA - Universidad de Salamanca Herbario; BC - Herbari BC, Institut Botànic de Barcelona..

Appendix S1b Samples of *Saxifraga spathularis* analysed

Country	Location	Code*	Lat (N)	Long (W)	*n*			
					*trn*S-G	Ss-C02	Ss-G04	Ss-G07
Ireland	Croaghaun Mountain	DBN-6031	53.9789	10.2047	1	1	1	1
	Lough Bunafreva	DBN-6033	54.0000	10.1803	2	1	2	2
	Streamstown	DBN-6010/6017/6018/6021	53.5267	10.0438	8	7	8	8
	Connemara National Park		53.5509	9.9454	4	4	4	4
	Dingle Peninsula		52.2168	9.9000	3	3	3	3
	Lough Cloon		51.9547	9.8653	8	6	8	8
	Caherconree	DBN-6020	52.2030	9.8537	1	-	1	1
	Ballaghbeama Gap		51.9432	9.8128	7	7	7	7
	Glancuttaun		52.045	9.775	8	2	8	8
	Leenaun		53.5972	9.7134	8	8	8	8
	Mamean		53.505	9.679	3	6	6	6
	Gap of Dunloe		52.0466	9.6500	8	7	8	8
	Galway Bridge		51.9665	9.5794	8	8	8	8
	Glengariff		51.7500	9.5667	8	6	8	8
	Lady Bantries Lookout		51.7415	9.5565	3	3	3	2
	Druidsview		51.80	9.55	8	8	8	8
	Nephin Mountain	DBN-6026	54.0138	9.3684	2	2	2	2
	Gougane Barra 1		51.8333	9.3450	8	8	8	8
	Gougane Barra 2		51.8340	9.2954	8	7	8	8
	Coomdorragh		51.8691	9.2658	8	8	8	8
	Burren		53.0352	9.0769	4	4	4	4
	Curragraig		52.1049	7.8657	8	8	8	8
	Mahon Falls		52.2324	7.5470	8	8	8	8

Appendix S1b (continued)

Country	Location	Code*	Lat (N)	Long (W)	*n*			
					*trn*S-G	Ss-C02	Ss-G04	Ss-G07
Portugal	Sierra de la Estrella	MGC-13014	40.3281	7.6327	1	1	-	1
Spain	Mazaricos	SANT-110427	42.9389	8.9922	4	4	4	4
	Los Peares	SALA-99560	42.36	7.79	1	1	1	1
	Cebreiro	SALA-115510	42.70	7.08	2	2	3	2
	La Coba	SALA-59413	43.21	6.97	3	3	3	3
	Peña Trevinca	FCO-15020	42,16	6.82	2	2	2	2
	Villar de Acero	LEB-48167	42.7286	6.8135	1	1	1	1
	Toreno	LEB-78833	42.7322	6.5044	1	1	-	1
	Cueto de Arbás	LEB-92470	42.9923	6.4299	4	4	4	4
	Villariño del Sil	LEB-17789	42.8737	6.4289	2	2	2	2
	Salas	FCO-29610	43.40	6.32	2	2	2	2
	Murias de Paredes	LEB-44527	42.7810	6.3105	1	1	2	3
	Pico la Berza	FCO-24445	43.18	6.21	2	2	1	2
	Caso	FCO-22519	43.10	5.33	3	3	3	3
	Pinar de Lillo	MGC-12919	43.0594	5.2832	3	3	3	3
	Puerto del Pontón	LEB-86557	43.0637	5.0878	2	2	2	2
	Portilla de la Reina	SALA-114146	43.04	4.85	4	4	4	3
	Polaciones	MGC-61618	43.04	4.29	1	1	1	1
					173	161	175	176

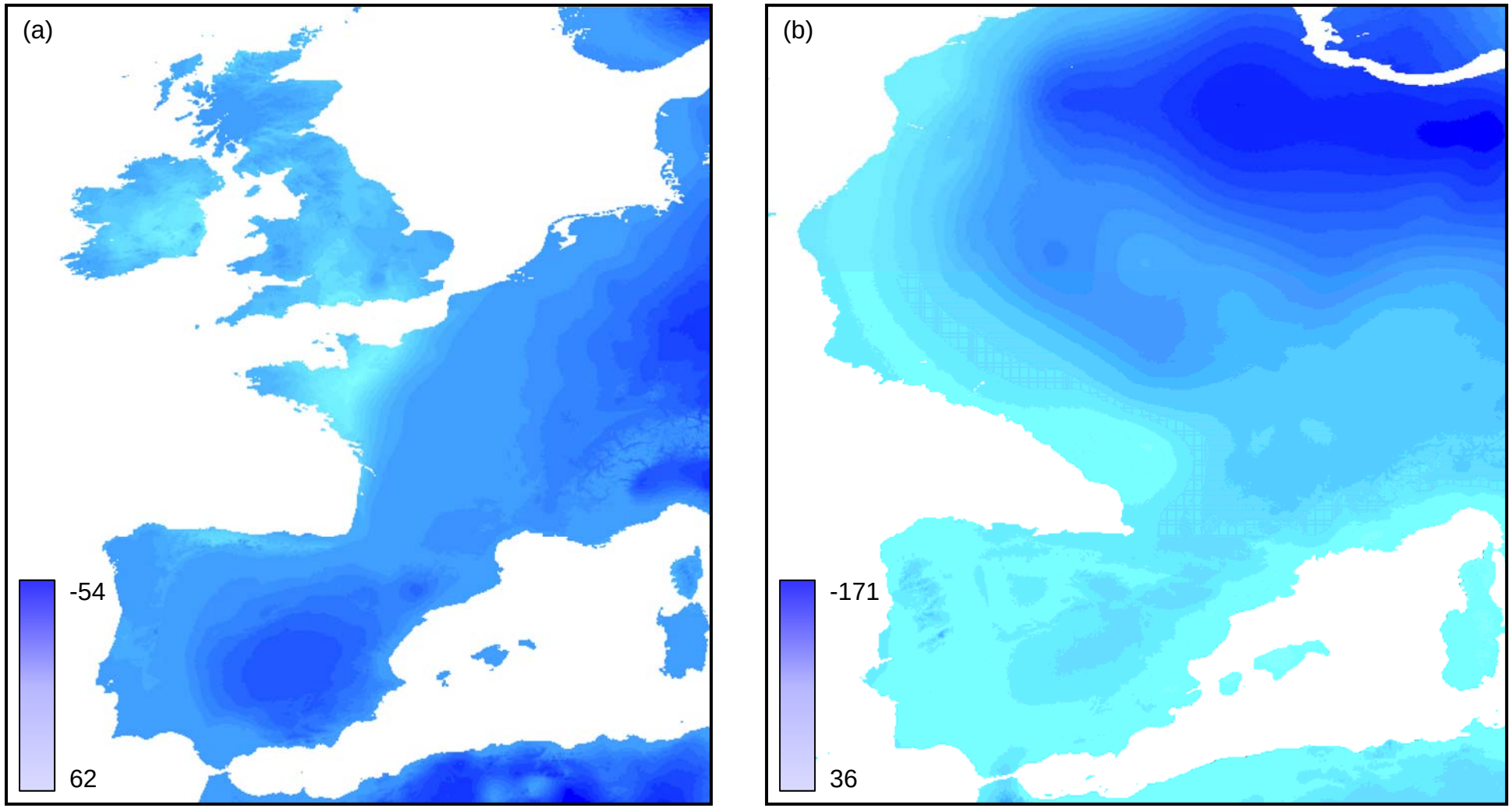
* Herbarium codes: DBN - National Botanic Gardens of Ireland, Glasnevin; MGC - Universidad de Málaga Herbario, SANT - Universidad de Santiago de Compostela Herbario; SALA - Universidad de Salamanca Herbario; FCO - Universidad de Oviedo Herbario; LEB - Universidad de León Herbario

Appendix S2 PCR / sequencing primers used in this study.

Species	Primer	Sequence (5' – 3')	Size (bp)[b]	Source
P. grandiflora	trnLF-F	GCTGTTCTAACAAATGGGGTTG	712	GenBank AF482623
	trnLF-R	CTGAGCTATCCCGACCATTC		
	trnLF-IN-R[a]	GAGAAACATTTTGGGAGTCAAATAG		
	trnLF-IN-F[a]	CTATTTGACTCCCAAAATGTTTCTC		
	Pg-C01-F	AGCAAGAGAAGGAAAATAAGAGTTT	206	This study
	Pg-C01-R	GCTCATAGACTACTGATACTTGAGCA		
	Pg-F02-F	TCTTGCATGGGTAGTTGGTG	400	This study
	Pg-F02-R	GTTGGCGTATGAAATTGTTGC		
	Pg-F10-F	ATAGGCCCGTGGCTGAAGT	292	This study
	Pg-F10-R	ACAATGGAATCCCCGACAG		
S. spathularis	trnSG-F	GCCGCTTTAGTCCACTCAGC	882-914	Hamilton (1999)
	trnSG-R	GAACGAATCACACTTTTACCAC		*Mol. Ecol.* **8**, 521-523
	trnSG-IN-R1[a]	GATAAACGTGATATATTTGTATC		
	trnSG-IN-F1[a]	GATACAAATATATCACGTTTATC		
	trnSG-IN-R2[a]	ATACTTGAATTCCCTATCATAG		
	trnSG-IN-F2[a]	CTATGATAGGGAATTCAAGTAT		
	Ss-C02-F	CACCCCATTACCTCATTCTTAGG	400 (84)	This study
	Ss-C02-R	TCGGCCACTATAAAGTTTTTCC		
	Ss-G04-F	TCCCTCTCTGAATAACACACGA	372 (95)	This study
	Ss-G04-R	TGGGAACGTAACCACAAACA		
	Ss-G07-F	CACGCCCCTAAAATAGAAGAAA	400 (211)	This study
	Ss-G07-R	ACGACTAAATCAACAATGGAGTC		

[a] Internal primer

[b] Figure in parenthesis indicates largest non-recombining portion used for analysis


(a)
-54
62
(b)
-171
36